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(54) Title: A METHOD FOR TRANSPLANTING CELLS INTO THE BRAIN AND THERAPEUTIC USES THEREFOR

(57) Abstract

A method for grafting a cell in the brain of a mammalian subject is accomplished by attaching the cell to a support matrix so that the cell attaches to the matrix outer surface, and implanting the support matrix with the attached cell into the brain. Preferred support matrices are glass or plastic microbeads having a diameter from about 90 to about 125 µm. The method employs cells of different types, preferably cells of neural or paraneural origin, such as adrenal chromaffin cells. Also useful are cell lines grown *in vitro*. Cells not of neural or paraneural origin, such as fibroblasts, may also be used following genetic alteration to express a desired neural product such as a neurotransmitter or a neuronal growth factor. The method is used to treat neurological diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy, and traumatic brain injury.

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A METHOD FOR TRANSPLANTING CELLS INTO THE BRAIN AND THERAPEUTIC USES THEREFOR

BACKGROUND OF THE INVENTION

5 Field of the Invention

The invention in the field of neuroscience and medicine relates to methods for implantation or transplantation of cells into the mammalian brain, useful in treating neurological disorders.

10 Description of the Background Art

Grafting of neural cells and neuron-like cells directly into the brain may be a practical approach to promote recovery from debilitating diseases of the nervous system such as Parkinson's disease (PD), Huntington's disease 15 (HD), Alzheimer's disease (AD), epilepsy, and familial dysautonomia, as well as injury or trauma to the nervous system. The characterization of factors which influence neurotransmitter phenotypic expression in cells placed into the brain may lead to a better understanding of processes 20 involved in normal specification in the nervous system and indicate means by which birth defects resulting from aberrant phenotypic expression can be therapeutically prevented or corrected.

Neurons or neuronal-like cells can be grafted into 25 the central nervous system (CNS), in particular, into the brain, either as solid tissue blocks or as dispersed cells. However, to date, a number of problems of both a technical and ethical nature have plagued the development of clinically feasible grafting procedures.

30 Parkinson's disease (PD) results from a selective loss of dopaminergic nigrostriatal neurons, resulting in a loss of input from the substantia nigra to the striatum. Solid grafts of tissues potentially capable of producing dopamine, such as adult adrenal medulla and embryonic substantia nigra 35 (SN), have been used extensively for experimental grafting in rats and primates treated with 6-hydroxydopamine (6-OHDA) to

destroy dopaminergic cells (Dunn tt, S.B. _et al._, _Brain Res._ _215_: 147-161 (1981); _ibid._ 229:457-470 (1981); Morisha, J.M. _et al._, _Exp. Neurol._ 84:643-654 (1984); Perlow, M.J. _et al._, _Science_ _204_:643-647 (1979)). Grafts of embryonic SN have
5 also been used as therapy for primates lesioned with the neurotoxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), which produces a PD-like disease (Redmond, D.E. _et al._, _Lancet_ 8490:1125-27 (1986)).

Stenevi _et al._ (_Brain Res._ _114_:1-20 (1976), in
10 investigating the optimal conditions for neural graft survival, found that the best results were obtained when (a) _fetal_ CNS neurons were used, and (b) the transplanted cells were placed next to a rich vascular supply. In fact, a review of the literature reveals that tissue from almost
15 every area of the fetal brain can be successfully transplan-ted if care is taken with procedural details (see, for example, Olson, L. A. _et al._, In: _Neural Transplants:_ _Development and Function_, Sladek, J.R. _et al._, eds, Plenum Press, New York, 1984, pp. 125-165).
20 Embryonic tissue provides an excellent source of cells which will differentiate in a foreign environment and become integrated with the host tissue. For example, grafts of embryonic SN into 6-OHDA treated rats have been shown to produce dopamine, to reduce apomorphine- or amphetamine-
25 induced rotation, to alleviate sensory deficits and to make synapses in the host striatum (Dunnett _et al._, Morisha _et_ _al._, Perlow _et al._, _supra_). Grafted neurons are also spontaneously active, thus mimicking normal adult SN neurons (Wuerthele, S.M. _et al._, In: _Catecholamines, Part B_, (E.
30 Usdin _et al._, eds.), A.R. Liss, Inc., New York, pp. 333-341).

In contrast to successful grafting of fetal neural tissue, mature CNS neurons have never been found to survive in transplants (Stenevi, U. _et al._, _Brain Res._ _114_:1-20 (1976)). The reason fetal CNS neurons survive grafting
35 procedures while adult neurons do not, while uncertain, is probably related to several factors. First, fetal neurons are less affected by low oxygen levels than mature neurons

(Jilek, L., In: <u>Developmental Neurobiology</u>, Himwich, W.A.,
ed., C.C. Thomas Publisher, Springfield, IL, 1970, pp. 331-
369), and grafting procedures necessarily involve periods of
anoxia until an adequate blood supply to the transplant is
5 established. Secondly, fetal neurons seem to survive best
when they are taken during a rapid growth phase and before
connections are established with target tissues (Boer, G.J.
<u>et al.</u>, <u>Neuroscience</u> <u>15</u>:1087-1109, (1985)). Also, fetal
tissue may be especially responsive to growth (or "survival")
10 factors which are known to be present in the milieu of the
damaged host brain (Nieto-Sampedro, M. <u>et al.</u>, <u>Science</u>
<u>217</u>:860-861 (1982); <u>Proc. Natl. Acad. Sci. USA</u> <u>81</u>:6250-6254
(1984)).

However, despite the promise of fetal tissue and
15 cell transplants, the art has turned to alternate sources of
donor tissues for transplantation because of the ethical,
moral, and legal problems attendant to utilizing fetal tissue
in human medicine. These sources include neural and
paraneural cells from organ donors and cultured cell lines.
20 (See, for example: Gash, D.M. <u>et al.</u>, In: <u>Neural Grafting in
the Mammalian CNS</u>, Bjorklund, A. <u>et al.</u>, eds, Elsevier,
Amsterdam, 1985, pp. 595-603; Gash, D.M. <u>et al.</u>, <u>Science</u>
<u>233</u>:1420-22 (1986)).

Although early clinical experiments using the
25 grafting approach did not result in long-lasting effects, an
initial report of one study appeared more promising (Madrazo
<u>et al.</u>, <u>Soc. Neurosci. Abstr.</u> <u>12</u>:563 (1986); for an overview,
see: Lieberman, A. <u>et al.</u>, <u>Adv. Tech. Stand. Neurosurg.</u>
<u>17</u>:65-76 (1990), which is hereby incorporated by reference).
30 Several additional observations suggest that grafting adrenal
cells should be a viable approach. Adrenal medullary cells
are derived from the neural crest and, like sympathetic
neurons, grow processes <u>in vivo</u> or <u>in vitro</u> in response to
nerve growth factor (NGF) (Unsicker, K. <u>et al.</u>, <u>Proc. Natl.</u>
35 <u>Acad. Sci. USA</u> <u>75</u>:3498-3502 (1978)). Solid grafts of adrenal
medulla from young rats can survive in the brain of 6-OHDA
treated rats for at least 5 months, produce dopamine and
reduce apomorphine induced rotation (Dunnett <u>et al.</u>, <u>supra</u>;

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Freed, W.J. <u>et al.</u>, <u>Ann. Neurol.</u> <u>8</u>:510-519 (1980); Freed,
W.J. <u>et al.</u> <u>Science</u> <u>222</u>:937-939 (1983)). These observations
suggest that given the appropriate environment, adrenal
medullary cells have the potential for growing catecholamine-
5 synthesizing fibers into brain tissue.

The potential for neuronal differentiation of
chromaffin cells is even better elucidated by grafts of
dissociated adrenal chromaffin cells which grow processes
when injected into rat striatum. Cultured adrenal medullary
10 cells also differentiate into neuronal-like cells with
processes when cultured in the presence of NGF (Unsicker,
<u>supra</u>). This remarkable plasticity of adrenal cells is
observed not only in morphology, but also in neurotransmitter
phenotypic expression. An array of neuropeptides including
15 vasoactive intestinal peptide, as well as the monoamine,
serotonin, are co-localized with catecholamines in adrenal
medullary cells (Schultzberg, M. <u>Neurosci.</u> <u>3</u>:1169-1186
(1978); Lundberg, J.M. <u>Proc. Natl. Acad. Sci. USA</u> <u>76</u>:4079-
4082 (1979)). Expression of these various phenotypes can be
20 modulated by extrinsic signals. For example, enkephalin and
VIP expression are increased following denervation of the
adrenal <u>in vivo</u>, by treatment of animals with nicotinic
blockers or after maintaining adrenal cells <u>in vitro</u>
(Tischler, A.S. <u>Life Sci.</u> <u>37</u>:1881-1886 (1985)). These
25 observations taken together with those of other studies
demonstrating that neurons derived from the neural crest can
switch phenotype during normal development (Bohn, M.C. <u>et
al.</u>, <u>Devel. Biol.</u> <u>82</u>:1-10 (1981); Jonakait, G.M. <u>et al.</u>
<u>Devel. Biol.</u> <u>88</u>:288-296 (1981)) or following experimental
30 manipulation of the micromilieu (LeDouarin, N.M. <u>Science</u> <u>231</u>:
1515-1522 (1986)) suggest that, in the future, it may be
possible to control the neurotransmitter phenotype expressed
by grafted cells either before and/or after grafting.

An additional advantage of grafting dissociated
35 cells compared to blocks of tissue is that the cells can be
precultured with various substances such as growth factors
prior to grafting or they can be co-grafted with other cells
or substances which promote specific parameters of differen-

tiation. Furtherm re, glial cells may hav specific regional effects and produce neuronal growth factors (Barbin, G. et al., Devel. Neurosci. 7:296-307 (1985); Schurch-Rathgeb, Y. et al., Nature 273:308-309 (1978); Unsicker, K. et al. Proc.
5 Natl. Acad. Sci. USA 81:2242-2246 (1984); Whitaker-Azmitia, P.M. et al., Brain Res. 497:80-85 (1989)). This suggests that co-transplanting cells providing the desired neurotrans-mitters along with specific types of glia which produce glial-derived factors, may promote neuronal growth and the
10 desired differentiation of grafted cells.

Thus, the feasibility of the transplant approach has been established experimentally. However, this approach is severely limited by the need for the use of fetal tissue, which is of limited availability and of great political
15 consequence. In essence, transplantation of human fetal tissue from aborted pregnancies has been prohibited in the United States. It would thus be of great benefit if simple, routine and safe methods for the successful transplantation of adult tissue into the brain were available for the
20 treatment of debilitating neurological disease.

Aebischer and his colleagues have successfully implanted selectively permeable biocompatible polymer capsules into the brain which encapsulated fragments of neural tissue which survived in this environment (Aebischer,
25 P. et al., Brain Res. 448:364-368 (1988); Winn, S.R. et al., J. Biomed Mater Res. 23:31-44 (1989). The polymer capsules consisted of polyvinyl chloride acrylic copolymer XM-50. Such permselective material completely prevented the invasion of the encapsulated tissue by host cells, and presumably
30 antibodies and viruses as well, based on the permeability of the membrane. When dopamine-releasing polymer rods were encapsulated into such a permselective polymer and implanted into denervated striatum in rats, alleviation of the experimentally induced Parkinson disease symptoms was
35 achieved (Winn S.R. et al., Exp. Neurol. 105:244-50 (1989). Furthermore, US Patent 4,892,538 (Aebischer et al., issued 1/9/90) discloses a cell culture device for implantation in a subject for delivery of a neurotransmitter comprising

secreting cells within a semipermeable membrane which permits diffusion of the neurotransmitter while excluding viruses, antibodies and other detrimental agents present in the external environment. The semipermeable membrane is of an

5 acrylic copolymer, polyvinylidene fluoride, polyurethane, polyalginate, cellulose acetal, polysulphone, polyvinyl alcohol, polyacrylonitrile, or their derivatives or mixtures and permits diffusion of solute of up to 50 kD molecular weight. This device is said to be useful in treatment of

10 neurotransmitter-deficient conditions, such as Parkinson's disease, by sustained, local delivery of neurotransmitters, precursors, agonists, fragments, etc., to a target area, especially the brain. The device may be made retrievable so that the contents may be renewed or supplemented, and the

15 cells are protected against immunological response and viral infection.

SUMMARY OF THE INVENTION

The inventors have made the unexpected discovery that by first culturing cells _in vitro_ on a support matrix,

20 such as 90 μm diameter glass beads, such cells including mature CNS neurons or cultured cells, can be successfully transplanted into the mammalian brain. The beads, to which the cells adhere, are stereotaxically injected into the recipient's brain. Cells so injected retain their viability

25 and are thus effectively transplanted. They show prolonged survival and viability _in vivo_, even when transplanted across species barriers.

It is an objective of the present invention to overcome the aforementioned deficiencies in the prior work.

30 The present invention provides a method for grafting a cell in the brain of a mammalian subject comprising culturing the cell with a support matrix so that the cell attaches to said matrix, and implanting the support matrix with the attached cell into the brain.

35 The method includes support matrices made of glass and other silicon oxides, polystyrene, polypropylene,

polyethylene, polyacrylamide, polycarbonate, polypentene,
acrylonitrile polymers, nylon, amylases, gelatin, collagen,
natural and modified polysaccharides, including dextrans and
celluloses (e.g. nitrocellulose), agar, and magnetite.
5 Preferred support matrices are beads, in particular micro-
beads having a diameter from about 90 to about 150 μm.

The method of the present invention may employ
cells of many different types, preferably either cells of
neural or paraneural origin, such as adrenal chromaffin
10 cells. Also useful are cell lines grown <u>in vitro</u>. Cells not
of neural or paraneural origin, such as fibroblasts, may also
be used following transfection with DNA encoding a neuropep-
tide or an enzyme or set of enzymes which results in
production of neurotransmitter, or a neuronal growth factor.
15   The present invention includes a method for
treating a neurological disease in a subject which comprises
grafting an effective number of cells capable of treating the
disease according to the above methods. Diseases which can
be treated according to the present invention include, but
20 are not limited to, Parkinson's disease, Alzheimer's
disease, Huntington's disease, epilepsy, familial dysauto-
nomia, and traumatic brain injury.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photomicrograph of a section of the
25 brain of a rat implanted with adult adrenomedullary cells on
a glass microbead. The section was fixed an stained
histochemically for tyrosine hydroxylase (TH) using horse
radish peroxidase. The darkly stained areas indicate cells
containing TH. The pattern of staining shows a needle tract
30 entering the section on the right side. The large circular
stained area in the center represents the bulk of the
implanted cells. Te injected cells form a "gland-like"
pattern, with some extending back into the needle tract.
(Thickness: 20 microns; magnification: 100x)

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is based on the unexpected discovery by the inventors that adult cells or cultured cells which cannot normally be transplanted into a mammalian brain
5 and survive, can be made to survive by first attaching them to a support matrix.

A number of different cell types are useful for the present invention. Typically, a cell will be selected based on its ability to provide a missing substance to the
10 recipient brain. Missing substances can be neurotransmitters or other neurally-active molecules, the absence of which results in neurological disease or dysfunction. It is important that the transplanted cell not grow as a tumor once it is inserted into the recipient.

15 The references cited below are all incorporated by reference herein, whether specifically incorporated or not.

By the term "neural or paraneural origin" is intended a cell which is derived from the embryonic neural crest. A preferred example of a cell of paraneural origin is
20 a adrenal medullary chromaffin cell. The precursor cells to the mammalian adrenal medulla are of neural crest origin and possess the potential to develop along either neuronal or endocrine lines of differentiation (Bohn, M.C. et al., 1981, supra, Devel. Biol. 89:299-308 (1982); Unsicker, K., Develop.
25 Biol. 108:259-268 (1985)). Chromaffin cells from the rat, monkey, and human adrenal medulla, when removed from adrenal cortical influences and exposed to nerve growth factor (NGF), change from an endocrine to a neuronal phenotype (Notter, M.F. et al., Cell Tiss. Res. 244:69-70 (1986); Stromberg, I.
30 et al., Exp. Brain Res. 60:335-349 (1985); Unsicker, K. et al., 1978, supra). When co-grafted with cerebral cortical or hippocampal tissue into the anterior chamber of the rat eye, adrenal chromaffin cells form nerve fibers which innervate the adjacent co-grafted brain tissue (Olson, L. A. et al.,
35 Exp. Neurol. 70414-426 (1980)). Another paraneural cell type is a retinal pigment epithelium cell (Song, M-K et al., J. Cell. Physiol. 148:196-203 (1990)).

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One source of donor c lls are established neural
cell lin s. Many neuronal clones exist which have b en used
extensively as model systems of development since they are
electrically active with appropriate surface receptors,
5 specific neurotransmitters, synapse forming properties and
the ability to differentiate morphologically and biochemical-
ly into normal neurons. Neural lines may express a tremen-
dous amount of genetic information which corresponds to the
genetic expression seen in CNS neurons. Such cells are
10 described in the following references: Kimhi, Y. et al.,
Proc. Natl. Acad. Sci. USA 73:462-466 (1976); In: Excitable
Cells in Tissue Culture, Nelson, P.G. et al., eds., Plenum
Press, New York, 1977, pp. 173-245); Prasad, K.M. et al., In:
Control of Proliferation of Animal Cells, Clarkson, B. et
15 al., eds., Cold Spring Harbor Laboratory Press, Cold Spring
Harbor, NY, 1974, pp. 581-594); Puro, D.G. et al., Proc.
Natl. Acad. Sci. USA 73:3544-3548 (1976); Notter, M.F. et
al., Devel. Brain Res. 26:59-68 (1986); Schubert, D. et al.,
Proc. Natl. Acad. Sci. USA 67:247-254 (1970); Kaplan, B.B. et
20 al., In: Basic and Clinical Aspects of Molecular Neurobiol-
ogy, Guffrida-Stella, A.M. et al., eds., Milano Fondozione
International Manarini, 1982)).

A major advantage of these cultured cells has been
the potential to manipulate the environment, as well as the
25 cells themselves, in controlling the phenotype and genotype.

For example, human neuroblastoma cells from the IMR
32 cell line can survive and express cholinergic markers in
primate brain nine months after transplantation (Gash, D.M.
et al., Science 233:1420-22 (1986)). These cells are
30 preferably treated to render them morphologically and
biochemically differentiated in vitro and must be rendered
permanently amitotic before implantation, which further aids
in their survival (Gash et al., supra; Gupta, M. et al., Dev.
Brain Res. 19:21-29 (1985)).
35 Whereas untreated pheochromocytoma/neuroblastoma
cells (PC12 cells) which grafted into the adult rat striatum
showed no survival after two w eks in vivo (Hefti, F. et al.,

<u>Brain Res.</u> <u>348</u>:283-288 (1985)), treatment <u>in vitro</u> with NGF
to stimulate neural cell differentiation followed by exposure
to antimitotic agents to inhibit cell proliferation, does not
deleteriously affect catecholamine neurotransmitter expres-
5 sion nor viability and is of value for stimulating perfor-
mance following transplant. Therefore, in one embodiment of
the present invention, cell line cells are modulated <u>in vitro</u>
with the appropriate growth or differentiation factor and
with an amitotic agent before transplantation in order to
10 promote cell survival and prevent expression of the malignant
potential.

It will be apparent to one of ordinary skill in the
art that examination of the neural cell surface of clonal
cells <u>in vitro</u> using conventional methods will permit the
15 selection of appropriate cells for use according to the
present invention. During normal development and differen-
tiation, the neuronal cell surface undergoes significant
changes which account for the migration, recognition and
integration of neurons in the CNS. For example, when mouse
20 C1300 clonal, neural cell lines are treated with antimitotic
agents or growth modulators such as prostaglandin E_1 and
cyclic AMP, new low molecular weight cell surface glycopro-
teins are produced (Bottenstein, J.E., In: <u>Functionally</u>
<u>Differentiated Cell Lines</u>, Sato, G.H., ed., Alan R. Liss, New
25 York, 1981). CNS surface gangliosides are induced which bind
tetanus toxin (Notter, M.F., 1986, <u>supra</u>) while specific
lectin binding glycoproteins appear which are similar to
normal central nervous system glycoproteins. Additionally,
receptors for neurotransmitters and neuropeptides on
30 neuroblastoma cells can be modified in a manner similar to
cells found in the CNS (Costa, L.G. <u>et al.</u>, <u>Biochem.</u>
<u>Pharmacol.</u> <u>91</u>:3407-3413 (1982).

Another important source of potential graft
material are cells engineered by somatic cell hybridization,
35 a process which can immortalize single neurons. Somatic cell
hybridization is a p werful c ll biologic tool used not only
to generate cell lines with a variety of genotypes but to
analyze the mechanisms r gulating the expression of various

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phenotypes of differentiation. Fusing cells which differ in
the expression of specific genes allows for the exploration
of the mechanisms controlling gene expression while chromo-
some alterations occur at rates to generate genetically

5   different cell lines. Hybrid cells can be formed which
retain the properties of differentiated cells. Hybrids
derived from fusion of sympathetic ganglia and neuroblastoma
cells can synthesize dopamine (Greene, L.A. et al., Proc.
Natl. Acad. Sci. USA 82:4923-4927 (1975) while brain cell

10 hybrids express choline acetyltransferase (Minna, J.D. et
al., Genetics 79:373-383 (1975)). Therefore, embryonic
precursors to dopaminergic neurons from the CNS can be fused
with mitotic cells to incorporate both genomes into a single
one that loses extra chromosomes over time and results in a

15 new hybrid line. It is within the skill of the art to
produce such hybrid neural or paraneural cells without undue
experimentation, screen them for the desired traits, such as
dopamine secretion, and select those having the best
potential for transplantation.

20   Another source of cells for transplantation
according to the present invention is the adrenal medulla.
This neural crest-derived tissue has been involved in
clinical trials (see Background) to treat PD. Adult monkey
adrenal medulla can be cultured in vitro for at least about

25 three weeks as single cells (Notter, M.F. et al., Cell Tiss.
Res. 244:69-76 (1986)). These cells respond to NGF by
phenotypic alteration from the epithelioid, glandular
morphology, to a neuronal morphology in which cells show
extensive neuritic arborizations containing microtubular

30 arrays. This neuronal phenotype appears to be critical for
long term survival of rat medullary cells in host CNS as well
as their integration with host tissue (Stromberg, L. et al.,
supra). Transplanted adrenal medulla tissue can correct
functional deficits resulting from nigrostriatal dopamine

35 d pletion in rats (see, for example, Freed et al., 1981,
supra). This, however, is thought to be brought about by
diffusion of dopamine from th transplant, a phenomenon that
decreases thr e to six months after transplantation. NGF

treatment of the transplanted cells induces fiber outgrowth from the transplant into the host and induces a longer lasting behavioral recovery (at least a year). Indeed, without NGF treatment, few chromaffin cells transplanted
5 either to rat (Freed et al., supra) or rhesus monkey caudate nucleus (Morihisia, J.M. et al., Exp. Neurol. 84:643-653 (1984) using prior art techniques survive. Similarly, retinal pigment epithelial cells secrete dopamine and other factors and may be used for brain implants according to the
10 present invention (Li, L. et al., Exp. Eye Res. 47:771-785 (1988); Lui, G.M. et al., Proc. Int'l. Soc. Eye Res. 6:172 (1990); Li, L. et al., Inv. Ophthal. Vis. Sci. 31(Suppl):595 (1990, abstr. 2915-13); Sheedlo, H.J. et al., ibid., abstr. 2916-14; Fektorovich, E.G. et al., ibid. (abstr. 2917-15);
15 Song, M-K et al., supra).

Cells transplanted into the mammalian brain according to the present invention have shown survival in the absence of added growth factors. However, an additional embodiment of the present invention is directed to transplan-
20 tation of cells attached to a support matrix combined with the treatment, either in vitro prior to transplant, in vivo after transplant, or both, with the appropriate growth/differentiation factor.

Human adrenal medullary cells can be maintained in
25 vitro having the neuronal phenotype for at least nine weeks (Notter, M.F. et al., Schmitt Neurol. Sci. Symp., June 30, 1987, abstr.). NGF induces this conversion from the glandular state. Adrenal medullary cells co-cultured with C6 glioma cells exhibit extensive neuritic arborization and
30 intimate contact with glioma cells. These astrocytic cells, treated with antimitotic agents to inhibit mitosis, are known to produce a growth factor similar to NGF which sustains sympathetic neurons in vitro (Barde, Y.A., Nature 274:818 (1978)). Grafted glial cells may play an important role in
35 functional recovery of neurons and may be an important source of trophic factors (Doering, L.C. et al., J. Neurolog. Sci. 63:183-196 (1984); Gumple, J. et al., Neurosci. Lett. 37: 307-311 (1984)). Therefore, another embodiment of the

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present inventi n involves co-culture of neural or paraneural
cells with glial cells, their co-incubation with a support
matrix, followed by implantation of the support matrix
carrying both cell types.

5   In additional embodiments of the present invention,
cells which are not of neural or paraneural origin, but which
have been altered to produce a substance of neurological
interest, are used. A preferred cell type is a human
foreskin fibroblast which is easily obtained and cultured,
10 and survives upon transplantation into the rat brain using
the methods of the present invention (see Example III). For
use in the present invention, the cells are genetically
altered, using methods known in the art, to express neuronal
growth factors, neurotransmitters, neuropeptides, or enzymes
15 involved in brain metabolism. (See, for example, Gage, F.H.
et al., Neuroscience 23:795-807 (1987); Rosenberg, M.B. et
al., Science 242:1575-1578 (1988); Shimohama, S. et al., Mol.
Brain Res. 5:271-278 (1989); which are hereby incorporated by
reference).

20   Standard reference works setting forth the general
principles of recombinant DNA technology and cell biology,
which are hereby incorporated by reference, include Watson,
J.D., et al., Molecular Biology of the Gene, Volumes I and
II, Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA
25 (1987); Darnell, J.E. et al., Molecular Cell Biology,
Scientific American Books, Inc., New York, NY (1986); Lewin,
B.M., Genes II, John Wiley & Sons, New York, NY (1985); Old,
R.W. et al., Principles of Gene Manipulation: An Introduction
to Genetic Engineering, 2nd Ed., University of California
30 Press, Berkeley, CA (1981); Maniatis, T., et al. (Molecular
Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold
Spring Harbor, NY (1982)); Sambrook, J. et al. (Molecular
Cloning: A Laboratory Manual, 2nd Edition, Cold Spring
Harbor Press, Cold Spring Harbor, NY (1989) and Albers, B. et
35 al., Molecular Biology of th Cell, 2nd Ed., Garland
Publishing, Inc., New York, NY (1989).

        The recombinant DNA molecules useful for the
methods of th pr s nt invention can b produced through any

of a variety of means, such as, for example, DNA or RNA
synthesis, or m re preferably, by application of recombinant
DNA techniques. Techniques for synthesizing such molecules
are disclosed by, for example, Wu, R., _et al._ (_Proc. Nucl._
5 _Acid. Res. Molec. Biol._ 21:101-141 (1978)). Procedures for
constructing recombinant molecules are disclosed in detail by
Sambrook _et al._, _supra_).

Typically, the gene or genes of interest are cloned
from a library of expression vectors which has been prepared
10 by cloning DNA or, more preferably, cDNA (from a cell capable
of expressing the gene) into an expression vector. The
library is then screened for members capable of expressing
the gene product of interest, such as a neurotransmitter-
synthesizing enzyme, using antibody binding with an antibody
15 specific for the gene product. DNA, or more preferably cDNA,
is extracted and purified from a cell which is capable of
expressing the gene product of interest. The purified cDNA
is fragmentized (by shearing, endonuclease digestion, etc.)
to produce a pool of DNA or cDNA fragments. DNA or cDNA
20 fragments from this pool are then cloned into an expression
vector in order to produce a library of expression vectors
whose members each contain a unique cloned DNA or cDNA
fragment.

An "expression vector" is a vector which (due to
25 the presence of appropriate transcriptional and/or transla-
tional control sequences) is capable of expressing a DNA (or
cDNA) molecule which has been cloned into the vector and of
thereby producing a polypeptide or protein. Expression of
the cloned sequences occurs when the expression vector is
30 introduced into an appropriate host cell. An appropriate
mammalian host cell would be any mammalian cell capable of
expressing the cloned sequences. Procedures for preparing
cDNA and for producing a genomic library are disclosed by
Sambrook _et al._ (_supra_).
35   A DNA sequence encoding a product r products the
expression of which is desired f r the present invention may
be recombined with vector DNA in accordance with conventional
techniques, including blunt-ended or staggered-ended termini

for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesiv ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

5 Techniques for such manipulations are disclosed by Sambrook et al., supra, and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and

10 translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene

15 expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the

20 DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

25 If desired, the non-coding region 3' to the gene sequence coding for the protein may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-

30 region naturally contiguous to the DNA sequence coding for the protein, the transcriptional termination signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be sub-

35 stituted.

Two DNA sequences (such as a promoter region sequence and a coding sequence) are said to be operably linked if the nature of the linkage between the two DNA

sequences does not (1) result in the introduction of a frameshift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the coding sequence, or (3) interfere with the ability of the
5 coding sequence to be transcribed by the promoter region sequence. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express the protein, transcriptional and translational signals recognized
10 by the host cell are necessary.

A promoter is a double-stranded DNA or RNA molecule which is capable of binding RNA polymerase and promoting the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the sequence of the
15 promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. A "promoter sequence complement" is a nucleic acid molecule whose sequence is the complement of a "promoter sequence." Hence, upon extension of a primer DNA or RNA adjacent to a single-
20 stranded "promoter sequence complement" or, of a "promoter sequence," a double-stranded molecule is created which will contain a functional promoter, if that extension proceeds towards the "promoter sequence" or the "promoter sequence complement." This functional promoter will direct the
25 transcription of a nucleic acid molecule which is operably linked to that strand of the double-stranded molecule which contains the "promoter sequence" (and not that strand of the molecule which contains the "promoter sequence complement").

The promoter sequences useful for producing cells
30 for the present invention may be either eukaryotic or viral. Suitable promoters are repressible, or, more preferably, constitutive. Useful eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, D., et al., J. Mol. Appl. Gen. 1:273-288 (1982)); the TK promoter of
35 Herpes virus (McKnight, S., Cell 31:355-365 (1982)); the SV40 early promoter (Benoist, C., et al., Nature (London) 290:304-310 (1981)). A preferred promoter, in particular for human fibroblasts, is the collagen promoter (Prockop, D.J. et al.,

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N. Eng. J. Med. 301:13-23, 77-85 (1979); Eyre, D.R., Science
207:1315-1322 (1980); Martin, G.R. et al., Trends Bioch. Sci.
10:285-287 (1985); which references are hereby incorporated
by reference).

5   Also intended within the scope of the present
invention are cells attached to a support matrix, according
to the invention, which are frozen and stored in a frozen
state using methods well known in the art. Following
thawing, the matrix-bound cells are implanted into a
10 recipient brain.

          The cells useful in the methods of the present
invention may be xenogeneic (= heterologous, i.e., derived
from a species different from the recipient), allogeneic (=
homologous, i.e., derived from a genetically different member
15 of the same species as the recipient) or autologous, wherein
the recipient also serves as the donor.

          The number of cells needed to achieve the purposes
of the present invention is variable depending on the size,
age, weight of the subject, the nature of the underlying
20 disease, other concurrent therapies, and the like. This can
be determined by one of skill in the art without undue
experimentation. In an adult human, an effective number of
cells attached to a support matrix are in the range of about
1×10^3 to about 1×10^7 cells, more preferably about 5×10^3
25 to about 1×10^6 cells. Alternatively, the effective amount
of transplanted cells can be determined in terms of mass of
cells added to a volume of beads, for example 40 mg of cell
mass per ml of beads.

          Materials of which the support matrix can be
30 comprised include those materials to which cells adhere
following in vitro incubation, and on which cells can grow,
and which can be implanted into a mammalian brain without
producing a toxic reaction, or an inflammatory or gliosis
reaction which would destroy the implanted cells or otherwise
35 interfere with their biological or therapeutic activity.
Such materials include, but are not limited to, glass and
other silicon oxides, polystyrene, polypropylene, polyethyl-
ene, polyvinylidene fluoride, polyurethane, polyalginate,

polysulphone, polyvinyl alcohol, acryl nitrile polymers, polyacrylamid , polycarbonate, polypentene, nylon, amylas s, gelatin, collagen, natural and modified polysaccharides, including dextrans and celluloses (e.g. nitrocellulose),
5 agar, and magnetite. Either resorbable or non-resorbable materials may be used.

The support matrix of the present invention is distinguished from that described by Aebischer and his colleagues (see above) in that, according to the present
10 invention, the cells are attached to the outer surface of the support; they are not encapsulated within a closed compartment, where their survival would be questionable given the exclusion capacity of the disclosed encapsulating supports. Furthermore, the support matrix of the present invention
15 presents no requirement that the material have particular permeability properties, such as the selective permeability of the Aebischer devices which allow low molecular weight substances to cross but exclude larger molecules (>50 kD).

The configuration of the support is preferably
20 spherical, as in a bead, but may be cylindrical, elliptical, a flat sheet or strip, a needle or pin shape, and the like. A preferred form of support matrix is a glass bead. Another preferred bead is a polystyrene bead. Bead sizes may range from about 10 μm to 1 cm in diameter, preferably from about
25 90 to about 150 μm. For a description of various microcarrier beads, see, for example, Fisher Biotech Source 87-88, Fisher Scientific Co., 1987, pp. 72-75; Sigma Cell Culture Catalog, Sigma Chemical Co., St. Louis, 1991, pp. 162-163; Ventrex Product Catalog, Ventrex Laboratories, 1989; these
30 references are hereby incorporated by reference. The upper limit on the bead size is dictated by the bead's stimulation of undesired host reactions such as gliosis, which may interfere with the function of the transplanted cells or cause damage to the surrounding brain tissue. Such limita-
35 tions are readily determinable by one of skill in the art.

To improve cell adhesion, survival and function, the solid matrix may optionally be coated on its external surface with factors known in the art to promote cell

adhesion, growth or survival. Such factors include cell adhesion molecules, extrac llular matrix, such as, for example, fibronectin, laminin, collagen, elastin, gycosamino-glycans, or proteoglycans (see: Albers, B. supra, pp. 802-
5 834) or growth factors, such as, for example, NGF. Alterna-tively, if the solid matrix to which the implanted cells are attached is constructed of porous material, the growth- or survival-promoting factor or factors may be incorporated into the matrix material, from which they would be slowly released
10 after implantation in vivo.

In an alternate embodiment of the present inven-tion, cells growing on resorbable matrices, such as, for example, collagen, can be implanted in sites of neurological interest other than the brain, in order to promote neuronal
15 regrowth or recovery. For example, cells attached to the matrix of the invention may be implanted into the spinal cord, or placed in or adjacent to the optic nerve.

The methods of the present invention are useful for treating a number of human neurological disease. Parkinson's
20 Disease can be treated according to the present invention by implanting dopamine-producing cells in the recipient's striatum. Alzheimer's disease (AD) involves a deficit in cholinergic cells in the nucleus basalis. Thus, according to the invention, a subject having AD or at risk therefor may be
25 implanted with cells producing acetylcholine.

Huntington's disease involves a gross wasting of the head of the caudate nucleus and putamen, usually accompanied by moderate disease of the gyrus. A subject suffering from Huntington's disease can be treated by
30 implanting cells producing the neurotransmitters gamma amino butyric acid (GABA), acetylcholine, or a mixture thereof. According to the present invention, the support matrix material to which such cells are attached is preferably implanted into the caudate and putamen.
35 Epilepsy is not truly a single disease but rather is a symptom produced by an underlying abnormality. One skilled in the art will appreciate that each epileptic subj ct will have damage or epileptic foci which are unique

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for the individual. Such foci can be localized using a
c mbination of diagnostic methods well-known in the art,
including electroencephalography, computerized axial
tomography and magnetic resonance imaging.

5   A patient suffering from epilepsy can be treated
according to the present invention by implanting the support
matrix material to which GABA-producing cells are attached
into the affected site. Since blockers of glutamate
receptors and NMDA receptors in the brain have been used to
10 control experimental epilepsy, cells producing molecules
which block excitatory amino acid pathways may be used
according to the invention. Thus implantation of cells
which have been modified as described herein to produce
polyamines, such as spermidine, in larger than normal
15 quantities may be useful for treating epilepsy.

          The methods of the present invention are intended
for use with any mammal which may experience the beneficial
effects of the methods of the invention. Foremost among such
mammals are humans, although the invention is not intended to
20 be so limited, and is also applicable to veterinary uses.

          Having now generally described the invention, the
same will be more readily understood through reference to the
following examples which are provided by way of illustration,
and are not intended to be limiting of the present invention,
25 unless specified.

                          EXAMPLE I

          Adrenal chromaffin cells from adult rats were
prepared according to known methods (see: Inoue, M. et al.,
A. J. Physiol. 257 (Cell Physiol. 26):C906-912 (1989)).
30 After sacrifice by nembutal anesthesia followed by decapita-
tion adrenal glands were removed. The medulla was freed of
capsular and cortical material by careful microdissection.
The tissue was cut into 2 or 3 pieces and incubated for 30
minutes with 0.2% collagenase in calcium-free balanced salt
35 solution containing 140 mM NaCl, 5 mM KCl, 5.2 mM $MgCl_2$, 5 mM
HEPES, 10 mM D-glucose, at pH 7.4. During the enzyme

treatment, the preparation was shaken gently by bubbling through 99.9% O_2. After dig stion, the tissue was washed three or four times with the above salt solution and then triturated gently in a pasteur pipet.

5   The dissociated chromaffin cells were transferred to cell culture flasks containing serum free medium (Ventrex PC 1) and were cultured overnight at 37°C in a 5% CO_2 atmosphere. Under these conditions, the cells retain their viability for no more than a few days. Sterilized glass

10 beads (Ventrex, 90 micron) were added to the culture flask. The cells attached to the beads and essentially formed a monolayer on the beads.   Within 24 hrs. of preparation in vitro, 1 to 5 μl aliquots of the cell-carrying bead suspension were injected into the brains of anesthetized

15 adult rats. Later analysis, at times when the cells would not have survived in culture, the cells were found to be viable in vivo in the site surrounding the injection, (see Figure 1) as determined by immunocytochemical staining for tyrosine hydroxylase according to the Weiner procedure (see:

20 Kilpatrick, D.L. et al., J. Neurochem. 35:679 (1980); Hokfelt, R. et al., Handbook of CHemical Neuroanatomy, Elsevier Science Publishers, Amsterdam (1985)). The glass beads produced no necrotic damage above that typical of the injection itself.

25   EXAMPLE II

          Adrenal medullae were removed from adult guinea pigs according to methods described in Example I and subjected to the procedure described in Example I. The cells on glass microbeads were injected into rat brain. The cells

30 survived for a period of at least 21 days in a completely healthy state. No signs of immunological rejection were evident.

                    EXAMPLE III

          Cultured human foreskin fibroblasts were attached

35 to glass microbeads as described above and injected into the rat brain. The cells were localized by immunofluorescence

using human IgG which selectively bound to the fibroblasts, followed by a fluorescein-conjugated goat anti-human IgG antibody. The implanted human cells survived for a period of at least 21 days in a completely healthy state. No signs of
5 immunological rejection were evident.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the
10 spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This
15 application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and
20 as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge,
25 readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is
30 to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

WHAT IS CLAIMED IS:

1. A method for grafting a cell in the brain of a mammalian subject comprising attaching said cell to the outer surface of a support matrix _in vitro_ and implanting said
5 support matrix with said attached cell into said brain.

2. The method of claim 1 wherein said support matrix is a microbead.

3. The method of claim 2 wherein said microbead has a diameter from about 90 to about 150 μm.

10 4. The method of claim 1 wherein said support matrix is made of a material selected from the group consisting of silicone oxide, polystyrene, polypropylene, polyethylene, polycarbonate, polypentene, acrylonitrile polymer, nylon, natural polysaccharide, modified polysac-
15 charide, amylase, collagen, natural cellulose, modified cellulose, agar and magnetite.

5. The method of claim 4 wherein said support matrix is silicon oxide.

6. The method of claim 1 wherein said cell is of
20 neural or paraneural origin.

7. The method of claim 6 wherein said cell is an adrenal chromaffin cell.

8. The method of claim 1 wherein said cell is a retinal pigment epithelial cell.

25 9. The method of claim 1 wherein said cell is from a cell line cultured _in vitro_.

10. The method of claim 9 wherein said cultured cell is a fibroblast.

11. The method of claim 9 wh rein said cultured cell has been genetically modified by transfection of genetic material encoding a protein or proteins rendering said genetically modified cell capable of producing a neurotrans-
5 mitter substance or a neuronal growth factor.

12. A method for treating a neurological disease in a subject comprising grafting into the brain of said subject an effective number of cells capable of treating said disease according to the method of claim 1.

10   13. The method of claim 12 wherein said disease is selected from a group consisting of Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy, and traumatic brain injury.

1/1

FIGURE 1

INTERNATIONAL SEARCH REPORT

International Application No.

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. Cl: 424/93,423,529,563,570,574; 435/240.23,240.24

IPC(5): A61K 37/00; C12N 5/00

II FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	424/93,423,433,520,529,563,570,574; 435/174,176,177,179,180,240.1,240.2,240.23,240.24; 604/891.1; 935/62.71

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

MEDLINE, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X / Y	US, A, 4,900,553 (Silver et al.) issued 13 February 1990, see the entire document.	1,4,6,7,9, 12,13
		2,3,5
Y	US, A, 4,748,121 (Beaver et al) issued 31 May 1988, see the entire document.	5
Y	Methods in Enzymology, Volume LVIII, Thilly et al., "Microcarrier Culture: A Homogeneous Environment for Studies of Cellular Biochemistry", pages 184-186, see the entire document. issued 1979,	1-13
Y	Science, Volume 242, issued 16 December 1988, Rosenberg el al., "Grafting Genetically Modified Cells to the Damaged Brain: Restorative Effects of NGF Expression," pages 1575-1577, see entire document.	1-13
Y -	US, A, 4,892,538 (Aebischer et al.) 09 January 1990, see entire document.	1-13

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

23 January 1992

Date of Mailing of this International Search Report

1 0 FEB 1992

International Searching Authority

ISA/US

Signature of Authorized Officer

Jacqueline M. Stone

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Published
With international search report.

(54) Title: A METHOD FOR TRANSPLANTING CELLS INTO THE BRAIN AND THERAPEUTIC USES THEREFOR

(57) Abstract

A method for grafting a cell in the brain of a mammalian subject is accomplished by attaching the cell to a support matrix so that the cell attaches to the matrix outer surface, and implanting the support matrix with the attached cell into the brain. Preferred support matrices are glass or plastic microbeads having a diameter from about 90 to about 125 μm. The method employs cells of different types, preferably cells of neural or paraneural origin, such as adrenal chromaffin cells. Also useful are cell lines grown *in vitro*. Cells not of neural or paraneural origin, such as fibroblasts, may also be used following genetic alteration to express a desired neural product such as a neurotransmitter or a neuronal growth factor. The method is used to treat neurological diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy, and traumatic brain injury.

A METHOD FOR TRANSPLANTING CELLS INTO THE BRAIN AND THERAPEUTIC USES THEREFOR

BACKGROUND OF THE INVENTION

5 Field of the Invention

The invention in the field of neuroscience and medicine relates to methods for implantation or transplantation of cells into the mammalian brain, useful in treating neurological disorders.

10 Description of the Background Art

Grafting of neural cells and neuron-like cells directly into the brain may be a practical approach to promote recovery from debilitating diseases of the nervous system such as Parkinson's disease (PD), Huntington's disease
15 (HD), Alzheimer's disease (AD), epilepsy, and familial dysautonomia, as well as injury or trauma to the nervous system. The characterization of factors which influence neurotransmitter phenotypic expression in cells placed into the brain may lead to a better understanding of processes
20 involved in normal specification in the nervous system and indicate means by which birth defects resulting from aberrant phenotypic expression can be therapeutically prevented or corrected.

Neurons or neuronal-like cells can be grafted into
25 the central nervous system (CNS), in particular, into the brain, either as solid tissue blocks or as dispersed cells. However, to date, a number of problems of both a technical and ethical nature have plagued the development of clinically feasible grafting procedures.
30   Parkinson's disease (PD) results from a selective loss of dopaminergic nigrostriatal neurons, resulting in a loss of input from the substantia nigra to the striatum. Solid grafts of tissues potentially capabl of producing dopamine, such as adult adrenal medulla and embryonic substantia nigra
35 (SN), have been used extensively for experimental grafting in rats and primates treated with 6-hydroxydopamine (6-OHDA) to

destroy dopaminergic cells (Dunnett, S.B. et al., Brain Res. 215: 147-161 (1981); ibid. 229:457-470 (1981); Morisha, J.M. et al., Exp. Neurol. 84:643-654 (1984); Perlow, M.J. et al., Science 204:643-647 (1979)). Grafts of embryonic SN have

5 also been used as therapy for primates lesioned with the neurotoxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), which produces a PD-like disease (Redmond, D.E. et al., Lancet 8490:1125-27 (1986)).

Stenevi et al. (Brain Res. 114:1-20 (1976), in

10 investigating the optimal conditions for neural graft survival, found that the best results were obtained when (a) fetal CNS neurons were used, and (b) the transplanted cells were placed next to a rich vascular supply. In fact, a review of the literature reveals that tissue from almost

15 every area of the fetal brain can be successfully transplanted if care is taken with procedural details (see, for example, Olson, L. A. et al., In: Neural Transplants: Development and Function, Sladek, J.R. et al., eds, Plenum Press, New York, 1984, pp. 125-165).

20 Embryonic tissue provides an excellent source of cells which will differentiate in a foreign environment and become integrated with the host tissue. For example, grafts of embryonic SN into 6-OHDA treated rats have been shown to produce dopamine, to reduce apomorphine- or amphetamine-

25 induced rotation, to alleviate sensory deficits and to make synapses in the host striatum (Dunnett et al., Morisha et al., Perlow et al., supra). Grafted neurons are also spontaneously active, thus mimicking normal adult SN neurons (Wuerthele, S.M. et al., In: Catecholamines, Part B, (E.

30 Usdin et al., eds.), A.R. Liss, Inc., New York, pp. 333-341).

In contrast to successful grafting of fetal neural tissue, mature CNS neurons have never been found to survive in transplants (Stenevi, U. et al., Brain Res. 114:1-20 (1976)). Th reason f tal CNS neurons survive grafting

35 procedures while adult neur ns do not, while uncertain, is probably related to several factors. First, fetal neurons are less affected by low oxygen levels than mature neurons

(Jilek, L., In: _Developmental Neurobiology_, Himwich, W.A., ed., C.C. Thomas Publisher, Springfield, IL, 1970, pp. 331-369), and grafting procedures necessarily involve periods of anoxia until an adequate blood supply to the transplant is
5 established. Secondly, fetal neurons seem to survive best when they are taken during a rapid growth phase and before connections are established with target tissues (Boer, G.J. et al., _Neuroscience_ 15:1087-1109, (1985)). Also, fetal tissue may be especially responsive to growth (or "survival")
10 factors which are known to be present in the milieu of the damaged host brain (Nieto-Sampedro, M. et al., _Science_ 217:860-861 (1982); _Proc. Natl. Acad. Sci. USA_ 81:6250-6254 (1984)).

However, despite the promise of fetal tissue and
15 cell transplants, the art has turned to alternate sources of donor tissues for transplantation because of the ethical, moral, and legal problems attendant to utilizing fetal tissue in human medicine. These sources include neural and paraneural cells from organ donors and cultured cell lines.
20 (See, for example: Gash, D.M. et al., In: _Neural Grafting in the Mammalian CNS_, Bjorklund, A. et al., eds, Elsevier, Amsterdam, 1985, pp. 595-603; Gash, D.M. et al., _Science_ 233:1420-22 (1986)).

Although early clinical experiments using the
25 grafting approach did not result in long-lasting effects, an initial report of one study appeared more promising (Madrazo et al., _Soc. Neurosci. Abstr._ 12:563 (1986); for an overvi w, see: Lieberman, A. et al., _Adv. Tech. Stand. Neurosurg._ 17:65-76 (1990), which is hereby incorporated by reference).
30 Several additional observations suggest that grafting adrenal cells should be a viable approach. Adrenal medullary cells are derived from the neural crest and, like sympathetic neurons, grow processes _in vivo_ or _in vitro_ in response to nerve growth factor (NGF) (Unsicker, K. et al., _Proc. Natl._
35 _Acad. Sci. USA_ 75:3498-3502 (1978)). Solid grafts of adrenal medulla from y ung rats can survive in the brain of 6-OHDA treat d rats for at least 5 months, produce dopamine and reduce apomorphine induced rotation (Dunnett et al., _supra_;

Freed, W.J. et al., Ann. Neurol. 8:510-519 (1980); Fr ed,
W.J. et al. Science 222:937-939 (1983)). These obs rvations
suggest that given the appropriate environment, adrenal
medullary cells have the potential for growing catecholamine-
5 synthesizing fibers into brain tissue.

The potential for neuronal differentiation of
chromaffin cells is even better elucidated by grafts of
dissociated adrenal chromaffin cells which grow processes
when injected into rat striatum. Cultured adrenal medullary
10 cells also differentiate into neuronal-like cells with
processes when cultured in the presence of NGF (Unsicker,
supra). This remarkable plasticity of adrenal cells is
observed not only in morphology, but also in neurotransmitter
phenotypic expression. An array of neuropeptides including
15 vasoactive intestinal peptide, as well as the monoamine,
serotonin, are co-localized with catecholamines in adrenal
medullary cells (Schultzberg, M. Neurosci. 3:1169-1186
(1978); Lundberg, J.M. Proc. Natl. Acad. Sci. USA 76:4079-
4082 (1979)). Expression of these various phenotypes can be
20 modulated by extrinsic signals. For example, enkephalin and
VIP expression are increased following denervation of the
adrenal in vivo, by treatment of animals with nicotinic
blockers or after maintaining adrenal cells in vitro
(Tischler, A.S. Life Sci. 37:1881-1886 (1985)). These
25 observations taken together with those of other studies
demonstrating that neurons derived from the neural crest can
switch phenotype during normal development (Bohn, M.C. et
al., Devel. Biol. 82:1-10 (1981); Jonakait, G.M. et al.
Devel. Biol. 88:288-296 (1981)) or following experimental
30 manipulation of the micromilieu (LeDouarin, N.M. Science 231:
1515-1522 (1986)) suggest that, in the future, it may be
possible to control the neurotransmitter phenotype expressed
by grafted cells either before and/or after grafting.

An additional advantage of grafting dissociated
35 cells compared to blocks f tissue is that the c lls can be
precultured with various substances such as growth factors
prior to grafting or they can be co-grafted with other cells
or substances which pr mote specific parameters of differen-

tiation. Furthermor , glial cells may have specific regional effects and produce neuronal growth factors (Barbin, G. et al., Devel. Neurosci. 7:296-307 (1985); Schurch-Rathgeb, Y. et al., Nature 273:308-309 (1978); Unsicker, K. et al. Proc.
5 Natl. Acad. Sci. USA 81:2242-2246 (1984); Whitaker-Azmitia, P.M. et al., Brain Res. 497:80-85 (1989)). This suggests that co-transplanting cells providing the desired neurotrans- mitters along with specific types of glia which produce glial-derived factors, may promote neuronal growth and the
10 desired differentiation of grafted cells.

Thus, the feasibility of the transplant approach has been established experimentally. However, this approach is severely limited by the need for the use of fetal tissue, which is of limited availability and of great political
15 consequence. In essence, transplantation of human fetal tissue from aborted pregnancies has been prohibited in th United States. It would thus be of great benefit if simple, routine and safe methods for the successful transplantation of adult tissue into the brain were available for the
20 treatment of debilitating neurological disease.

Aebischer and his colleagues have successfully implanted selectively permeable biocompatible polymer capsules into the brain which encapsulated fragments of neural tissue which survived in this environment (Aebischer,
25 P. et al., Brain Res. 448:364-368 (1988); Winn, S.R. et al., J. Biomed Mater Res. 23:31-44 (1989). The polymer capsules consisted of polyvinyl chloride acrylic copolymer XM-50. Such permselective material completely prevented the invasion of the encapsulated tissue by host cells, and presumably
30 antibodies and viruses as well, based on the permeability of the membrane. When dopamine-releasing polymer rods were encapsulated into such a permselective polymer and implanted into denervated striatum in rats, alleviation of the experimentally induced Parkinson disease symptoms was
35 achieved (Winn S.R. et al., Exp. Neurol. 105:244-50 (1989). Furtherm re, US Patent 4,892,538 (Aebischer et al., issued 1/9/90) discloses a cell culture devic for implantation in a subject for delivery of a neur transmitter comprising

secr ting c lls within a semipermeable membrane which permits diffusion of the neurotransmitter while excluding viruses, antibodies and other detrimental agents present in the external environment. The semipermeable membrane is of an
5 acrylic copolymer, polyvinylidene fluoride, polyurethane, polyalginate, cellulose acetal, polysulphone, polyvinyl alcohol, polyacrylonitrile, or their derivatives or mixtures and permits diffusion of solute of up to 50 kD molecular weight. This device is said to be useful in treatment of
10 neurotransmitter-deficient conditions, such as Parkinson's disease, by sustained, local delivery of neurotransmitters, precursors, agonists, fragments, etc., to a target area, especially the brain. The device may be made retrievable so that the contents may be renewed or supplemented, and the
15 cells are protected against immunological response and viral infection.

SUMMARY OF THE INVENTION

The inventors have made the unexpected discovery that by first culturing cells in vitro on a support matrix,
20 such as 90 μm diameter glass beads, such cells including mature CNS neurons or cultured cells, can be successfully transplanted into the mammalian brain. The beads, to which the cells adhere, are stereotaxically injected into the recipient's brain. Cells so injected retain their viability
25 and are thus effectively transplanted. They show prolonged survival and viability in vivo, even when transplanted across species barriers.

It is an objective of the present invention to overcome the aforementioned deficiencies in the prior work.
30   The present invention provides a method for grafting a cell in the brain of a mammalian subject comprising culturing the cell with a support matrix so that the cell attaches to said matrix, and implanting the support matrix with the attach d cell into the brain.
35   The method includes support matrices made of glass and other silicon oxides, polystyren , polypropylene,

p lyethylene, polyacrylamide, polycarbonate, polypentene, acrylonitrile polymers, nylon, amylases, gelatin, collagen, natural and modified polysaccharides, including dextrans and celluloses (e.g. nitrocellulose), agar, and magnetite.

5 Preferred support matrices are beads, in particular microbeads having a diameter from about 90 to about 150 μm.

The method of the present invention may employ cells of many different types, preferably either cells of neural or paraneural origin, such as adrenal chromaffin

10 cells. Also useful are cell lines grown in vitro. Cells not of neural or paraneural origin, such as fibroblasts, may also be used following transfection with DNA encoding a neuropeptide or an enzyme or set of enzymes which results in production of neurotransmitter, or a neuronal growth factor.

15 The present invention includes a method for treating a neurological disease in a subject which comprises grafting an effective number of cells capable of treating the disease according to the above methods. Diseases which can be treated according to the present invention include, but

20 are not limited to, Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy, familial dysautonomia, and traumatic brain injury.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photomicrograph of a section of the

25 brain of a rat implanted with adult adrenomedullary cells on a glass microbead. The section was fixed an stained histochemically for tyrosine hydroxylase (TH) using horse radish peroxidase. The darkly stained areas indicate cells containing TH. The pattern of staining shows a needle tract

30 entering the section on the right side. The large circular stained area in the center represents the bulk of the implanted cells. Te injected cells form a "gland-like" pattern, with some extending back into the needle tract. (Thickness: 20 microns; magnification: 100x)

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is based on the unexpected discovery by the inventors that adult cells or cultured cells which cannot normally be transplanted into a mammalian brain 5 and survive, can be made to survive by first attaching them to a support matrix.

A number of different cell types are useful for the present invention. Typically, a cell will be selected based on its ability to provide a missing substance to the 10 recipient brain. Missing substances can be neurotransmitters or other neurally-active molecules, the absence of which results in neurological disease or dysfunction. It is important that the transplanted cell not grow as a tumor once it is inserted into the recipient.

15 The references cited below are all incorporated by reference herein, whether specifically incorporated or not.

By the term "neural or paraneural origin" is intended a cell which is derived from the embryonic neural crest. A preferred example of a cell of paraneural origin is 20 a adrenal medullary chromaffin cell. The precursor cells to the mammalian adrenal medulla are of neural crest origin and possess the potential to develop along either neuronal or endocrine lines of differentiation (Bohn, M.C. et al., 1981, supra, Devel. Biol. 89:299-308 (1982); Unsicker, K., Develop. 25 Biol. 108:259-268 (1985)). Chromaffin cells from the rat, monkey, and human adrenal medulla, when removed from adrenal cortical influences and exposed to nerve growth factor (NGF), change from an endocrine to a neuronal phenotype (Notter, M.F. et al., Cell Tiss. Res. 244:69-70 (1986); Stromberg, I. 30 et al., Exp. Brain Res. 60:335-349 (1985); Unsicker, K. et al., 1978, supra). When co-grafted with cerebral cortical or hippocampal tissue into the anterior chamber of the rat eye, adrenal chromaffin cells form nerve fibers which innervate the adjacent co-grafted brain tissue (Olson, L. A. et al., 35 Exp. Neurol. 70414-426 (1980)). Another paran ural cell type is a retinal pigment epithelium cell (Song, M-K et al., J. Cell. Physiol. 148:196-203 (1990)).

One source of don r cells are established neural
c ll lines. Many neuronal clones exist which have been used
extensively as model systems of development since they are
electrically active with appropriate surface receptors,
5 specific neurotransmitters, synapse forming properties and
the ability to differentiate morphologically and biochemical-
ly into normal neurons. Neural lines may express a tremen-
dous amount of genetic information which corresponds to the
genetic expression seen in CNS neurons. Such cells are
10 described in the following references: Kimhi, Y. et al.,
Proc. Natl. Acad. Sci. USA 73:462-466 (1976); In: Excitable
Cells in Tissue Culture, Nelson, P.G. et al., eds., Plenum
Press, New York, 1977, pp. 173-245); Prasad, K.M. et al., In:
Control of Proliferation of Animal Cells, Clarkson, B. et
15 al., eds., Cold Spring Harbor Laboratory Press, Cold Spring
Harbor, NY, 1974, pp. 581-594); Puro, D.G. et al., Proc.
Natl. Acad. Sci. USA 73:3544-3548 (1976); Notter, M.F. et
al., Devel. Brain Res. 26:59-68 (1986); Schubert, D. et al.,
Proc. Natl. Acad. Sci. USA 67:247-254 (1970); Kaplan, B.B. et
20 al., In: Basic and Clinical Aspects of Molecular Neurobiol-
ogy, Guffrida-Stella, A.M. et al., eds., Milano Fondozione
International Manarini, 1982)).

A major advantage of these cultured cells has been
the potential to manipulate the environment, as well as the
25 cells themselves, in controlling the phenotype and genotype.

For example, human neuroblastoma cells from the IMR
32 cell line can survive and express cholinergic markers in
primate brain nine months after transplantation (Gash, D.M.
et al., Science 233:1420-22 (1986)). These cells are
30 preferably treated to render them morphologically and
biochemically differentiated in vitro and must be rendered
permanently amitotic before implantation, which further aids
in their survival (Gash et al., supra; Gupta, M. et al., Dev.
Brain Res. 19:21-29 (1985)).

35 Whereas untreated pheochromocytoma/neuroblastoma
cells (PC12 cells) which grafted into the adult rat striatum
showed no survival after two we ks in vivo (Hefti, F. et al.,

Brain Res. 348:283-288 (1985)), treatment in vitro with NGF
t stimulate neural cell differentiation followed by exposure
to antimitotic agents to inhibit cell proliferation, does not
deleteriously affect catecholamine neurotransmitter expres-
5 sion nor viability and is of value for stimulating perfor-
mance following transplant. Therefore, in one embodiment of
the present invention, cell line cells are modulated in vitro
with the appropriate growth or differentiation factor and
with an amitotic agent before transplantation in order to
10 promote cell survival and prevent expression of the malignant
potential.

It will be apparent to one of ordinary skill in the
art that examination of the neural cell surface of clonal
cells in vitro using conventional methods will permit the
15 selection of appropriate cells for use according to the
present invention. During normal development and differen-
tiation, the neuronal cell surface undergoes significant
changes which account for the migration, recognition and
integration of neurons in the CNS. For example, when mouse
20 C1300 clonal, neural cell lines are treated with antimitotic
agents or growth modulators such as prostaglandin E_1 and
cyclic AMP, new low molecular weight cell surface glycopro-
teins are produced (Bottenstein, J.E., In: Functionally
Differentiated Cell Lines, Sato, G.H., ed., Alan R. Liss, New
25 York, 1981). CNS surface gangliosides are induced which bind
tetanus toxin (Notter, M.F., 1986, supra) while specific
lectin binding glycoproteins appear which are similar to
normal central nervous system glycoproteins. Additionally,
receptors for neurotransmitters and neuropeptides on
30 neuroblastoma cells can be modified in a manner similar to
cells found in the CNS (Costa, L.G. et al., Biochem.
Pharmacol. 91:3407-3413 (1982).

Another important source of potential graft
material are cells engineered by somatic cell hybridization,
35 a process which can immortaliz singl neur ns. Somatic cell
hybridization is a powerful cell biologic t ol used not only
to generate cell lines with a variety of genotypes but to
analyze the mechanisms regulating the expression of various

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phenotypes of differentiation. Fusing cells which differ in
the expression of specific genes allows for the exploration
of the mechanisms controlling gene expression while chromo-
some alterations occur at rates to generate genetically
5   different cell lines. Hybrid cells can be formed which
retain the properties of differentiated cells. Hybrids
derived from fusion of sympathetic ganglia and neuroblastoma
cells can synthesize dopamine (Greene, L.A. et al., Proc.
Natl. Acad. Sci. USA 82:4923-4927 (1975) while brain cell
10 hybrids express choline acetyltransferase (Minna, J.D. et
al., Genetics 79:373-383 (1975)). Therefore, embryonic
precursors to dopaminergic neurons from the CNS can be fused
with mitotic cells to incorporate both genomes into a single
one that loses extra chromosomes over time and results in a
15 new hybrid line. It is within the skill of the art to
produce such hybrid neural or paraneural cells without undue
experimentation, screen them for the desired traits, such as
dopamine secretion, and select those having the best
potential for transplantation.
20   Another source of cells for transplantation
according to the present invention is the adrenal medulla.
This neural crest-derived tissue has been involved in
clinical trials (see Background) to treat PD. Adult monkey
adrenal medulla can be cultured in vitro for at least about
25 three weeks as single cells (Notter, M.F. et al., Cell Tiss.
Res. 244:69-76 (1986)). These cells respond to NGF by
phenotypic alteration from the epithelioid, glandular
morphology, to a neuronal morphology in which cells show
extensive neuritic arborizations containing microtubular
30 arrays. This neuronal phenotype appears to be critical for
long term survival of rat medullary cells in host CNS as well
as their integration with host tissue (Stromberg, L. et al.,
supra). Transplanted adrenal medulla tissue can correct
functional deficits resulting from nigrostriatal dopamine
35 depleti n in rats (see, f r example, Fr ed et al., 1981,
supra). This, however, is thought to b brought about by
diffusion of dopamine from the transplant, a phenomenon that
decreases three to six months after transplantation. NGF

treatment of th transplanted cells induces fiber outgrowth from the transplant into th host and induces a long r lasting behavioral recovery (at least a year). Indeed, without NGF treatment, few chromaffin cells transplanted
5 either to rat (Freed et al., supra) or rhesus monkey caudate nucleus (Morihisia, J.M. et al., Exp. Neurol. 84:643-653 (1984) using prior art techniques survive. Similarly, retinal pigment epithelial cells secrete dopamine and other factors and may be used for brain implants according to the
10 present invention (Li, L. et al., Exp. Eye Res. 47:771-785 (1988); Lui, G.M. et al., Proc. Int'l. Soc. Eye Res. 6:172 (1990); Li, L. et al., Inv. Ophthal. Vis. Sci. 31(Suppl):595 (1990, abstr. 2915-13); Sheedlo, H.J. et al., ibid., abstr. 2916-14; Fektorovich, E.G. et al., ibid. (abstr. 2917-15);
15 Song, M-K et al., supra).

Cells transplanted into the mammalian brain according to the present invention have shown survival in the absence of added growth factors. However, an additional embodiment of the present invention is directed to transplan-
20 tation of cells attached to a support matrix combined with the treatment, either in vitro prior to transplant, in vivo after transplant, or both, with the appropriate growth/dif- ferentiation factor.

Human adrenal medullary cells can be maintained in
25 vitro having the neuronal phenotype for at least nine weeks (Notter, M.F. et al., Schmitt Neurol. Sci. Symp., June 30, 1987, abstr.). NGF induces this conversion from the glandular state. Adrenal medullary cells co-cultured with C6 glioma cells exhibit extensive neuritic arborization and
30 intimate contact with glioma cells. These astrocytic cells, treated with antimitotic agents to inhibit mitosis, are known to produce a growth factor similar to NGF which sustains sympathetic neurons in vitro (Barde, Y.A., Nature 274:818 (1978)). Grafted glial cells may play an important role in
35 functional recovery of neurons and may be an important source of trophic factors (Doering, L.C. et al., J. Neurolog. Sci. 63:183-196 (1984); Gumple, J. et al., Neurosci. Lett. 37: 307-311 (1984)). Therefore, an ther embodiment of the

present invention involves co-culture of neural or paran ural
cells with glial cells, th ir co-incubation with a support
matrix, followed by implantation of the support matrix
carrying both cell types.

5   In additional embodiments of the present invention,
cells which are not of neural or paraneural origin, but which
have been altered to produce a substance of neurological
interest, are used. A preferred cell type is a human
foreskin fibroblast which is easily obtained and cultured,
10 and survives upon transplantation into the rat brain using
the methods of the present invention (see Example III). For
use in the present invention, the cells are genetically
altered, using methods known in the art, to express neuronal
growth factors, neurotransmitters, neuropeptides, or enzymes
15 involved in brain metabolism. (See, for example, Gage, F.H.
et al., Neuroscience 23:795-807 (1987); Rosenberg, M.B. et
al., Science 242:1575-1578 (1988); Shimohama, S. et al., Mol.
Brain Res. 5:271-278 (1989); which are hereby incorporated by
reference).

20   Standard reference works setting forth the general
principles of recombinant DNA technology and cell biology,
which are hereby incorporated by reference, include Watson,
J.D., et al., Molecular Biology of the Gene, Volumes I and
II, Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA
25 (1987); Darnell, J.E. et al., Molecular Cell Biology,
Scientific American Books, Inc., New York, NY (1986); Lewin,
B.M., Genes II, John Wiley & Sons, New York, NY (1985); Old,
R.W. et al., Principles of Gene Manipulation: An Introduction
to Genetic Engineering, 2nd Ed., University of California
30 Press, Berkeley, CA (1981); Maniatis, T., et al. (Molecular
Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold
Spring Harbor, NY (1982)); Sambrook, J. et al. (Molecular
Cloning: A Laboratory Manual, 2nd Edition, Cold Spring
Harbor Press, Cold Spring Harbor, NY (1989) and Albers, B. et
35 al., Molecular Biology of the Cell, 2nd Ed., Garland
Publishing, Inc., New York, NY (1989).

        The recombinant DNA molecules useful for th
methods of the pr sent invention can be produced through any

of a variety of means, such as, for exampl , DNA or RNA
synthesis, or more preferably, by application of recombinant
DNA techniques. Techniques for synthesizing such molecules
are disclosed by, for example, Wu, R., et al. (Prog. Nucl.
5 Acid. Res. Molec. Biol. 21:101-141 (1978)). Procedures for
constructing recombinant molecules are disclosed in detail by
Sambrook et al., supra).

Typically, the gene or genes of interest are cloned
from a library of expression vectors which has been prepared
10 by cloning DNA or, more preferably, cDNA (from a cell capable
of expressing the gene) into an expression vector. The
library is then screened for members capable of expressing
the gene product of interest, such as a neurotransmitter-
synthesizing enzyme, using antibody binding with an antibody
15 specific for the gene product. DNA, or more preferably cDNA,
is extracted and purified from a cell which is capable of
expressing the gene product of interest. The purified cDNA
is fragmentized (by shearing, endonuclease digestion, etc.)
to produce a pool of DNA or cDNA fragments. DNA or cDNA
20 fragments from this pool are then cloned into an expression
vector in order to produce a library of expression vectors
whose members each contain a unique cloned DNA or cDNA
fragment.

An "expression vector" is a vector which (due to
25 the presence of appropriate transcriptional and/or transla-
tional control sequences) is capable of expressing a DNA (or
cDNA) molecule which has been cloned into the vector and of
thereby producing a polypeptide or protein. Expression of
the cloned sequences occurs when the expression vector is
30 introduced into an appropriate host cell. An appropriate
mammalian host cell would be any mammalian cell capable of
expressing the cloned sequences. Procedures for preparing
cDNA and for producing a genomic library are disclosed by
Sambrook et al. (supra).
35 A DNA sequence encoding a product or products the
expression of which is desired for the present invention may
be recombined with vector DNA in accordance with conventional
techniques, including blunt-ended or staggered-ended termini

for ligation, restriction enzyme digestion to provide
appropriate termini, filling in of coh sive ends as
appropriate, alkaline phosphatase treatment to avoid
undesirable joining, and ligation with appropriate ligases.

5   Techniques for such manipulations are disclosed by Sambrook
et al., supra, and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be
"capable of expressing" a polypeptide if it contains
nucleotide sequences which contain transcriptional and

10 translational regulatory information and such sequences are
"operably linked" to nucleotide sequences which encode the
polypeptide. An operable linkage is a linkage in which the
regulatory DNA sequences and the DNA sequence sought to be
expressed are connected in such a way as to permit gene

15 expression. The precise nature of the regulatory regions
needed for gene expression may vary from organism to
organism, but shall in general include a promoter region
which, in prokaryotes, contains both the promoter (which
directs the initiation of RNA transcription) as well as the

20 DNA sequences which, when transcribed into RNA, will signal
the initiation of protein synthesis. Such regions will
normally include those 5'-non-coding sequences involved with
initiation of transcription and translation, such as the TATA
box, capping sequence, CAAT sequence, and the like.

25   If desired, the non-coding region 3' to the gene
sequence coding for the protein may be obtained by the above-
described methods. This region may be retained for its
transcriptional termination regulatory sequences, such as
termination and polyadenylation. Thus, by retaining the 3'-

30 region naturally contiguous to the DNA sequence coding for
the protein, the transcriptional termination signals may be
provided. Where the transcriptional termination signals are
not satisfactorily functional in the expression host cell,
then a 3' region functional in the host cell may be sub-

35 stituted.

Two DNA sequences (such as a promoter region
sequence and a coding sequence) are said to be operably
linked if the nature of the linkage betw en the two DNA

sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the coding sequence, or (3) interfere with the ability of the
5 coding sequence to be transcribed by the promoter region sequence. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express the protein, transcriptional and translational signals recognized
10 by the host cell are necessary.

     A promoter is a double-stranded DNA or RNA molecule which is capable of binding RNA polymerase and promoting the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the sequence of the
15 promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. A "promoter sequence complement" is a nucleic acid molecule whose sequence is the complement of a "promoter sequence." Hence, upon extension of a primer DNA or RNA adjacent to a single-
20 stranded "promoter sequence complement" or, of a "promoter sequence," a double-stranded molecule is created which will contain a functional promoter, if that extension proceeds towards the "promoter sequence" or the "promoter sequence complement." This functional promoter will direct the
25 transcription of a nucleic acid molecule which is operably linked to that strand of the double-stranded molecule which contains the "promoter sequence" (and not that strand of the molecule which contains the "promoter sequence complement").

     The promoter sequences useful for producing cells
30 for the present invention may be either eukaryotic or viral. Suitable promoters are repressible, or, more preferably, constitutive. Useful eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen.* 1:273-288 (1982)); the TK promoter of
35 Herpes virus (McKnight, S., *Cell* 31:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature (London)* 290:304-310 (1981)). A preferred promoter, in particular for human fibroblasts, is the collagen promoter (Prockop, D.J. *et al.*,

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N. Eng. J. Med. 301:13-23, 77-85 (1979); Eyre, D.R., Science
207:1315-1322 (1980); Martin, G.R. et al., Trends Bioch. Sci.
10:285-287 (1985); which references are hereby incorporated
by reference).

5   Also intended within the scope of the present
invention are cells attached to a support matrix, according
to the invention, which are frozen and stored in a frozen
state using methods well known in the art. Following
thawing, the matrix-bound cells are implanted into a
10 recipient brain.

        The cells useful in the methods of the present
invention may be xenogeneic (= heterologous, i.e., derived
from a species different from the recipient), allogeneic (=
homologous, i.e., derived from a genetically different member
15 of the same species as the recipient) or autologous, wherein
the recipient also serves as the donor.

        The number of cells needed to achieve the purposes
of the present invention is variable depending on the size,
age, weight of the subject, the nature of the underlying
20 disease, other concurrent therapies, and the like. This can
be determined by one of skill in the art without undue
experimentation. In an adult human, an effective number of
cells attached to a support matrix are in the range of about
1×10^3 to about 1×10^7 cells, more preferably about 5×10^3
25 to about 1×10^6 cells. Alternatively, the effective amount
of transplanted cells can be determined in terms of mass of
cells added to a volume of beads, for example 40 mg of cell
mass per ml of beads.

        Materials of which the support matrix can be
30 comprised include those materials to which cells adhere
following in vitro incubation, and on which cells can grow,
and which can be implanted into a mammalian brain without
producing a toxic reaction, or an inflammatory or gliosis
reaction which would destroy the implanted cells or otherwise
35 interfere with their biological or th rapeutic activity.
Such materials include, but ar not limited to, glass and
other silicon oxides, polystyrene, polypropylene, polyethyl-
ene, polyvinylidene fluoride, polyur thane, polyalginate,

polysulphone, polyvinyl alc hol, acrylonitrile polymers, polyacrylamid , polycarbonate, polypentene, nylon, amylases, gelatin, collagen, natural and modified polysaccharides, including dextrans and celluloses (e.g. nitrocellulose),
5 agar, and magnetite. Either resorbable or non-resorbable materials may be used.

The support matrix of the present invention is distinguished from that described by Aebischer and his colleagues (see above) in that, according to the present
10 invention, the cells are attached to the outer surface of the support; they are not encapsulated within a closed compartment, where their survival would be questionable given the exclusion capacity of the disclosed encapsulating supports. Furthermore, the support matrix of the present invention
15 presents no requirement that the material have particular permeability properties, such as the selective permeability of the Aebischer devices which allow low molecular weight substances to cross but exclude larger molecules (>50 kD).

The configuration of the support is preferably
20 spherical, as in a bead, but may be cylindrical, elliptical, a flat sheet or strip, a needle or pin shape, and the like. A preferred form of support matrix is a glass bead. Another preferred bead is a polystyrene bead. Bead sizes may range from about 10 μm to 1 cm in diameter, preferably from about
25 90 to about 150 μm. For a description of various microcarrier beads, see, for example, Fisher Biotech Source 87-88, Fisher Scientific Co., 1987, pp. 72-75; Sigma Cell Culture Catalog, Sigma Chemical Co., St. Louis, 1991, pp. 162-163; Ventrex Product Catalog, Ventrex Laboratories, 1989; these
30 references are hereby incorporated by reference. The upper limit on the bead size is dictated by the bead's stimulation of undesired host reactions such as gliosis, which may interfere with the function of the transplanted cells or cause damage to the surrounding brain tissue. Such limita-
35 tions are readily determinable by one f skill in the art.

To improve c ll adhesion, survival and function, the solid matrix may optionally b coated on its external surface with factors known in the art to promote cell

adh sion, gr wth or survival. Such factors include cell
adhesion molecules, extracellular matrix, such as, for
example, fibronectin, laminin, collagen, elastin, gycosamino-
glycans, or proteoglycans (see: Albers, B. supra, pp. 802-
5 834) or growth factors, such as, for example, NGF. Alterna-
tively, if the solid matrix to which the implanted cells are
attached is constructed of porous material, the growth- or
survival-promoting factor or factors may be incorporated into
the matrix material, from which they would be slowly released
10 after implantation in vivo.

In an alternate embodiment of the present inven-
tion, cells growing on resorbable matrices, such as, for
example, collagen, can be implanted in sites of neurological
interest other than the brain, in order to promote neuronal
15 regrowth or recovery. For example, cells attached to the
matrix of the invention may be implanted into the spinal
cord, or placed in or adjacent to the optic nerve.

The methods of the present invention are useful for
treating a number of human neurological disease. Parkinson's
20 Disease can be treated according to the present invention by
implanting dopamine-producing cells in the recipient's
striatum. Alzheimer's disease (AD) involves a deficit in
cholinergic cells in the nucleus basalis. Thus, according to
the invention, a subject having AD or at risk therefor may be
25 implanted with cells producing acetylcholine.

Huntington's disease involves a gross wasting of
the head of the caudate nucleus and putamen, usually
accompanied by moderate disease of the gyrus. A subject
suffering from Huntington's disease can be treated by
30 implanting cells producing the neurotransmitters gamma amino
butyric acid (GABA), acetylcholine, or a mixture thereof.
According to the present invention, the support matrix
material to which such cells are attached is preferably
implanted into the caudate and putamen.

35   Epilepsy is not truly a single disease but rather
is a symptom produced by an underlying abnormality. One
skilled in the art will appreciate that each epileptic
subject will have damage or epileptic foci which are unique

treatm nt, the pr paration was shaken gently by bubbling through 99.9% O_2. After digestion, the tissue was washed three or four times with the above salt solution and then triturated gently in a pasteur pipet.

5   The dissociated chromaffin cells were transferred to cell culture flasks containing serum free medium (Ventrex PC 1) and were cultured overnight at 37°C in a 5% CO_2 atmosphere. Under these conditions, the cells retain their viability for no more than a few days. Sterilized glass
10 beads (Ventrex, 90 micron) were added to the culture flask. The cells attached to the beads and essentially formed a monolayer on the beads.   Within 24 hrs. of preparation in vitro, 1 to 5 μl aliquots of the cell-carrying bead suspension were injected into the brains of anesthetized
15 adult rats. Later analysis, at times when the cells would not have survived in culture, the cells were found to be viable in vivo in the site surrounding the injection, (see Figure 1) as determined by immunocytochemical staining for tyrosine hydroxylase according to the Weiner procedure (see:
20 Kilpatrick, D.L. et al., J. Neurochem. 35:679 (1980); Hokfelt, R. et al., Handbook of CHemical Neuroanatomy, Elsevier Science Publishers, Amsterdam (1985)). The glass beads produced no necrotic damage above that typical of the injection itself.

25   EXAMPLE II
     Adrenal medullae were removed from adult guinea pigs according to methods described in Example I and subjected to the procedure described in Example I. The cells on glass microbeads were injected into rat brain. The cells
30 survived for a period of at least 21 days in a completely healthy state. No signs of immunological rejection were evident.

                              EXAMPLE III
     Cultur d human foreskin fibroblasts were attached
35 t glass microbeads as described above and injected into the rat brain. The cells were localized by immunofluorescence

using human IgG which selectively bound to the fibroblasts,
followed by a fluor scein-conjugat d goat anti-human IgG
antibody. The implanted human cells survived for a period of
at least 21 days in a completely healthy state. No signs of
5 immunological rejection were evident.

        Having now fully described this invention, it will
be appreciated by those skilled in the art that the same can
be performed within a wide range of equivalent parameters,
concentrations, and conditions without departing from the
10 spirit and scope of the invention and without undue ex-
perimentation.

        While this invention has been described in
connection with specific embodiments thereof, it will be
understood that it is capable of further modifications. This
15 application is intended to cover any variations, uses, or
adaptations of the inventions following, in general, the
principles of the invention and including such departures
from the present disclosure as come within known or customary
practice within the art to which the invention pertains and
20 as may be applied to the essential features hereinbefore set
forth as follows in the scope of the appended claims.

        The foregoing description of the specific embodi-
ments will so fully reveal the general nature of the
invention that others can, by applying current knowledge,
25 readily modify and/or adapt for various applications such
specific embodiments without departing from the generic
concept, and, therefore, such adaptations and modifications
should and are intended to be comprehended within the meaning
and range of equivalents of the disclosed embodiments. It is
30 to be understood that the phraseology or terminology employed
herein is for the purpose of description and not of limita-
tion.

WHAT IS CLAIMED IS:

1. A method for grafting a cell in the brain of a mammalian subject comprising attaching said cell to the outer surface of a support matrix _in vitro_ and implanting said
5 support matrix with said attached cell into said brain.

2. The method of claim 1 wherein said support matrix is a microbead.

3. The method of claim 2 wherein said microbead has a diameter from about 90 to about 150 μm.

10 4. The method of claim 1 wherein said support matrix is made of a material selected from the group consisting of silicone oxide, polystyrene, polypropylene, polyethylene, polycarbonate, polypentene, acrylonitrile polymer, nylon, natural polysaccharide, modified polysac-
15 charide, amylase, collagen, natural cellulose, modified cellulose, agar and magnetite.

5. The method of claim 4 wherein said support matrix is silicon oxide.

6. The method of claim 1 wherein said cell is of
20 neural or paraneural origin.

7. The method of claim 6 wherein said cell is an adrenal chromaffin cell.

8. The method of claim 1 wherein said cell is a retinal pigment epithelial cell.

25 9. The method of claim 1 wherein said cell is from a cell line cultured _in vitro_.

10. The method of claim 9 wherein said cultur d cell is a fibroblast.

11. The method of claim 9 wherein said cultured cell has been genetically modified by transfection of genetic material encoding a protein or proteins rendering said genetically modified cell capable of producing a neurotrans-
5 mitter substance or a neuronal growth factor.

12. A method for treating a neurological disease in a subject comprising grafting into the brain of said subject an effective number of cells capable of treating said disease according to the method of claim 1.

10   13. The method of claim 12 wherein said disease is selected from a group consisting of Parkinson's disease, Alzheimer's disease, Huntington's disease, epilepsy, and traumatic brain injury.

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07443

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. Cl: 424/93,423,529,563,570,574; 435/240.23,240.24
IPC(5): A61K 37/00; C12N 5/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/93,423,433,520,529,563,570,574; 435/174,176,177,179,180,240.1,240.2,240.23,240.24; 604/891.1; 935/62.71

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

MEDLINE, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
X / Y	US, A, 4,900,553 (Silver et al.) issued 13 February 1990, see the entire document.	1,4,6,7,9, 12,13 / 2,3,5
Y	US, A, 4,748,121 (Beaver et al) issued 31 May 1988, see the entire document.	5
Y	Methods in Enzymology, Volume LVIII, issued 1979, Thilly et al., "Microcarrier Culture: A Homogeneous Environment for Studies of Cellular Biochemistry", pages 184-186, see the entire document.	1-13
Y	Science, Volume 242, issued 16 December 1988, Rosenberg el al., "Grafting Genetically Modified Cells to the Damaged Brain: Restorative Effects of NGF Expression," pages 1575-1577, see entire document.	1-13
Y -	US, A, 4,892,538 (Aebischer et al.) 09 January 1990, see entire document.	1-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

23 January 1992

Date of Mailing of this International Search Report

10 FEB 1992

International Searching Authority

ISA/US

Signature of Authorized Officer

Jacqueline M. Stone

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

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